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### **► To cite this version:**

Abir Tadmouri, Shigeki Kiyonaka, Maud Barbado, Matthieu Rousset, Katell Fablet, et al.. Cacnb4 directly couples electrical activity to gene expression, a process defective in juvenile epilepsy.. EMBO Journal, 2012, 31 (18), pp.3730-44. 10.1038/emboj.2012.226 . inserm-00757285

**HAL Id: inserm-00757285**

**<https://www.hal.inserm.fr/inserm-00757285>**

Submitted on 1 Feb 2013

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# **Cacnb4 directly couples electrical activity to gene expression, a process defective in juvenile epilepsy**

Running title: *Cacnb4, an adaptor protein for gene regulation*

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**Calcium current through voltage-gated calcium channels (VGCC) controls gene expression. Here, we describe a novel signaling pathway in which the VGCC Cacnb4 subunit directly couples neuronal excitability to transcription. Electrical activity induces Cacnb4 association to Ppp2r5d, a regulatory subunit of PP2A phosphatase, followed by i) nuclear translocation of Cacnb4/Ppp2r5d/PP2A, ii) association with the tyrosine hydroxylase (TH) gene promoter through the nuclear transcription factor thyroid hormone receptor alpha (TR $\alpha$ ), and iii) histone binding through association of Cacnb4 with HP1 $\gamma$  concomitantly with Ser<sup>10</sup> histone H3 dephosphorylation by PP2A. This signaling cascade leads to TH gene repression by Cacnb4 and is controlled by the state of interaction between the SH3 and guanylate kinase (GK) modules of Cacnb4. The human R482X CACNB4 mutation, responsible for a form of juvenile myoclonic epilepsy, prevents association with Ppp2r5 and nuclear targeting of the complex by altering Cacnb4 conformation. These findings demonstrate that an intact VGCC subunit acts as a repressor recruiting platform to control neuronal gene expression.**

*Keywords:*  $\beta$ 4 subunit / Gene regulation / thyroid receptor alpha / phosphatase 2A / HP1 $\gamma$

## **Introduction**

Voltage-gated calcium channels (VGCC) are heteromultimeric complexes that translate electric signals into calcium influx (Catterall et al, 2005), thereby controlling synaptic vesicle exocytosis, neuronal excitability and gene expression (Deisseroth et al, 2003; Flavell & Greenberg, 2008; Greer & Greenberg, 2008). In VGCC, the pore-forming subunit is associated to auxiliary subunits, among which the cytoplasmic Cacnb4

( $\beta_4$ ) plays an essential function in channel expression level at the plasma membrane and biophysical properties (Arikkath & Campbell, 2003). Mutations in the genes encoding VGCC induce diverse neuronal pathologies, such as epilepsy, ataxia, autism, and migraine (Bidaud et al, 2006). In humans, a mutation of *CACNB4*, leading to a 38 amino acid truncation of  $\beta_4$  C-terminus, has been associated to juvenile myoclonic epilepsy (Escayg et al, 2000). In mice, a four-nucleotide insertion into a splice donor site of *Cacnb4* results in a truncation of 60% of the protein sequence and a *lethargic (lh)* phenotype (Burgess et al, 1997). How these mutations may affect VGCC-mediated function still remains an open question. Calcium entering through VGCC has been implicated in gene regulation by activating calcium-binding proteins that propagate the signal to the nucleus (Dolmetsch et al, 2001; Graef et al, 1999; Oliveria et al, 2007; Rosen et al, 1994) or by diffusing to the nucleus with or without signal amplification by intracellular stores (Carrion et al, 1999; Hardingham et al, 2001; Hardingham et al, 1997). Evidences for alternative pathways have been reported. First, an atypical short  $\beta_4$  splice variant,  $\beta_{4c}$ , was shown to interact with heterochromatin protein 1 gamma (HP1 $\gamma$ ), a nuclear protein involved in gene silencing and transcription regulation (Hibino et al, 2003; Xu et al, 2011). However,  $\beta_{4c}$  lacks the domain required for association with the pore subunit (Chen et al, 2004) and therefore cannot couple neuronal activity to gene regulation. A second pathway implicates CCAT, a C-terminal fragment of *Cacna1c* VGCC, as a calcium-regulated transcription factor (Gomez-Ospina et al, 2006). In this case, calcium influx triggers CCAT nuclear export providing a calcium-dependent link between neuronal activity and transcription. The origin of this fragment (channel proteolysis or alternative splicing) remains unknown.

Here, we show that  $\beta_4$  directly couples neuronal excitability to gene expression along a signaling pathway that is disrupted by the human R482X mutation. We found that electrical activity promotes the formation of a new nuclear complex in which  $\beta_4$  plays the role of an organizing platform that brings together a transcription factor for DNA binding, a phosphatase for histone dephosphorylation and HP1 $\gamma$  for nucleosome association. Formation of this complex controls gene activity as witnessed by the case of tyrosine hydroxylase (TH) gene.

## Results

### *$\beta_4$ nuclear localization*

$\beta_4$  is highly expressed in the hippocampal dentate gyrus of adult wild-type (wt) C57Bl/6 mice brain but not in *lh* mice brain (Figure 1a).  $\beta_4$  labeling is strongest in soma and coincides with NeuN nuclear labeling, suggesting that a fraction of  $\beta_4$  protein may be localized in cell nuclei, in agreement with previous observations (Subramanyam et al, 2009). Western blot analyses of wt brain lysates show a unique 58 kDa band, corresponding to  $\beta_4$ , absent in *lh* (Figure 1b). Western blot analyses of cytoplasmic *versus* nuclear fractions of wt brain lysates show the presence of  $\beta_4$  in the nucleus (Figure 1c). Electron microscopy (EM) demonstrates the presence of  $\beta_4$  in the nucleus of CA1 hippocampal neurons (Figure 1d & Supplementary Figure 1). In primary cultures of hippocampal neurons,  $\beta_4$  is predominantly expressed within the cytoplasm/plasma membrane at 5 DIV (Figure 1e, left panels). In contrast, the protein is densely located in the nucleus at 18 DIV (Figure 1e, right panels). The nuclear to cytoplasmic density ratios (NCR values) of  $\beta_4$  increases as a function of time from 0.07 (3 DIV) to 3.81 (18 DIV) on

average indicating the differentiation-dependent progressive targeting of  $\beta_4$  to neuronal nuclei (Figure 1f). NCR value increase is accompanied by a histogram broadening witnessing higher  $\beta_4$  subcellular distribution variability among neurons.  $\beta_4$  nuclear appearance coincides with that of VGCC currents and electrical activity at 5 DIV (unpublished observation). EM analysis of hippocampal neurons at 5 and 18 DIV also illustrate the increase of  $\beta_4$  nuclear density at 18 DIV (Figure 1g). Synchronized neuronal differentiation of NG108-15 cells, induced by serum deprivation and 1 mM cAMP, is accompanied by VGCC expression (Chemin et al, 2002). In undifferentiated NG108.15 cells,  $\beta_4$  is exclusively cytoplasmic, while present in nuclei after 13 days of differentiation (Figure 1h). Mean NCR values illustrate that nuclear  $\beta_4$  expression is triggered by neuronal differentiation (Figure 1i).

#### ***$\beta_4$ epilepsy mutation alters nuclear targeting***

To search for a  $\beta_4$  nuclear targeting domain, truncated  $\beta_4$ -EGFP constructs were expressed in CHO cells (Figure 2a). In CHO and HEK293 cells,  $\beta_4$ -EGFP spontaneously locates in the nucleus (NCR =  $10.1 \pm 3.3$  for CHO cells). In contrast, truncated constructs are deficient in nuclear targeting suggesting that  $\beta_4$  has no nuclear localization sequence (NLS) or that the NLS sequence depends on  $\beta_4$  structure integrity (Figure 2b,c). Indeed, five constructs display a distribution similar to EGFP alone (NCR  $\sim 1$ ). Two constructs, that contain  $\beta_4$  guanylate kinase (GK) domain, show preferential cytoplasm distribution (NCR =  $0.27 \pm 0.2$  and  $0.26 \pm 0.2$  for  $\beta_{216-402}$ -EGFP and  $\beta_{216-519}$ -EGFP, respectively).  $\beta_4$ -EGFP truncation therefore results in 10 to 40-fold decrease of nuclear targeting efficiency. These data were confirmed for two truncated  $\beta_4$  constructs by Western blot

analyses of nuclear and cytoplasmic fractions of transfected HEK293 cells (Supplementary Figure 2a). An earlier report mentioned the importance of a restricted N-terminal domain for  $\beta_4$  nuclear accumulation (Subramanyam et al, 2009). As observed in this study, mutation of this domain significantly reduced nuclear accumulation albeit by a limited extent (by 1.41-fold, from a mean NCR value of  $11.4 \pm 0.7$  to  $8.1 \pm 0.7$ ). However,  $\beta_{R28A-R29A-S30A}$ -EGFP still significantly accumulates into the nucleus in contrast to the constructs presented in this manuscript.

We engineered a  $\beta_{1-481}$ -EGFP mutant (Figure 2a) corresponding to the human juvenile myoclonic epilepsy mutation (Escayg et al, 2000) and assessed its cellular distribution in CHO cells (Figure 2b,c). Nuclear accumulation of  $\beta_{1-481}$ -EGFP mutant is strongly reduced (NCR =  $0.98 \pm 0.62$ ; 10.3-fold lower than  $\beta_4$ -EGFP NCR). This mutation induces a loss in nuclear targeting as shown by Western blot analyses of nuclear and cytoplasmic fractions of CHO cells (Figure 2d). Finally,  $\beta_{1-481}$ -EGFP mutant is completely excluded from hippocampal neuron nuclei at 7 DIV (NCR =  $0.32 \pm 0.18$ ; Figure 2e,f). This corresponds to a 9.5-fold decrease in NCR value compared to  $\beta_4$ -EGFP ( $3.15 \pm 1.76$ ), a reduction similar to that measured in CHO cells. Double imaging of immunolabeled endogenous  $\beta_4$  and  $\beta_{1-481}$ -EGFP indicates the extent of nuclear exclusion of the mutant (Figure 2g). In conclusion, the nuclear targeting of  $\beta_4$  requires the preservation of its structural integrity. The human epilepsy mutation alters this structural integrity and hinders nuclear targeting.

### ***The SH3/GK interaction controls $\beta_4$ nuclear targeting***

Interaction between  $\beta$  SH3 and GK domains regulates channel activity (McGee et al, 2004; Takahashi et al, 2005). Its role was therefore investigated on  $\beta_4$  nuclear targeting. Three constructs were designed:  $\beta_{1-166}$ -EGFP,  $\beta_{200-519}$ -myc, and  $\beta_{200-481}$ -myc (corresponding to the juvenile epilepsy deletion) (Figure 3a). In agreement with Figure 2c, none of these constructs show a specific nuclear targeting in CHO cells (Figure 3b). Expressed together,  $\beta_{1-166}$ -EGFP and  $\beta_{200-519}$ -myc reconstitute a nuclear-targeting-competent complex (Figure 3c; NCR =  $6.9 \pm 1.6$  for  $\beta_{1-166}$ -EGFP, and  $7.1 \pm 1.1$  for  $\beta_{200-519}$ -myc). In contrast, expression of  $\beta_{1-166}$ -EGFP with  $\beta_{200-481}$ -myc fails to form a nuclear complex (Figure 3c, right panels). Co-immunoprecipitation experiments show that  $\beta_{200-481}$ -myc no longer interacts with  $\beta_{1-166}$ -EGFP contrary to  $\beta_{200-519}$ -myc (Figure 3d). These data suggest that the human mutation prevents  $\beta_4$  nuclear targeting by modulating the SH3/GK interaction. This issue was further addressed by investigating the effect of two mutations (SH3 L125P or GK P225R) known to disrupt the SH3/GK interaction (McGee et al, 2004; Takahashi et al, 2004) (Figure 3e). As shown,  $\beta_{1-166}$ -L125P-EGFP no longer interacts with  $\beta_{200-519}$ -myc (Figure 3f). Also, both  $\beta_{L125P}$ -EGFP and  $\beta_{P225R}$ -EGFP are completely excluded from hippocampal neuron nuclei (Figure 3g). NCR values,  $0.43 \pm 0.15$  (L125P) and  $0.43 \pm 0.11$  (P225R), correspond to a 7.3-fold decrease in mutant  $\beta_4$  nuclear localization (Figure 3h). These results demonstrate that  $\beta_4$  nuclear targeting requires the internal SH3/GK and reveal a previously unrecognized structural role of the last C-terminal 38 amino acid sequence in modulating the interaction between the C-terminal containing hemi- $\beta_4$  subunit and the SH3-containing  $\beta_4$  fragment.

***The phosphatase 2A Ppp2r5d (B56 $\delta$ ) subunit contributes to  $\beta_4$  nuclear localization***



Absence of a clear molecular determinant for  $\beta_4$  nuclear targeting, suggests that this process requires at least one protein partner, whereas defective  $\beta_{1-481}$ ,  $\beta_{L125P}$  and  $\beta_{P225R}$  nuclear localization indicates that interaction with this (these) partners depends on SH3/GK interaction state. Yeast two-hybrid screenings were performed with a mouse brain complementary DNA library using  $\beta_4$  as bait. The 62 positive clones were subjected to two-hybrid  $\beta$ -galactosidase assays using  $\beta_4$  and  $\beta_{1-481}$  as baits. B56 $\delta$ , a 594 amino acid protein containing a poorly-defined NLS at its C-terminus, was revealed to interact with  $\beta_4$  but not with  $\beta_{1-481}$  (Figure 4a). B56 $\delta$  is one of the two regulatory subunits of phosphatase 2A (PP2A), along with B56 $\gamma$ , known to target PP2A to the nucleus (McCright et al, 1996). Co-immunoprecipitation confirms that  $\beta_4$ -EGFP, but not  $\beta_{1-481}$ -EGFP, interacts with B56 $\delta$ -myc expressed in HEK293 cells (Figure 4b). As expected, Cacna1e and RIM1 were also identified as  $\beta_4$  partners (Kiyonaka et al, 2007). In contrast to B56 $\delta$ , both proteins interact equally well with  $\beta_4$  and  $\beta_{1-481}$ . These yeast two-hybrid assays also reveal that Cacnb3 ( $\beta_3$ ), another  $\beta$  isoform, also interacts with B56 $\delta$  suggesting the existence of redundant signaling pathways (Figure 4c).  $\beta_{4c}$ , a short  $\beta_4$  splice variant, known to interact with the HP1 $\gamma$  chromo shadow domain (Hibino et al, 2003), does not interact with B56 $\delta$ . Conversely,  $\beta_4$  does not interact with HP1 $\gamma$  in these conditions (Figure 4c). Truncated  $\beta_4$  constructs were analyzed for B56 $\delta$  interaction by yeast two-hybrid assays (Figure 4d).  $\beta_{49-519}$  is the only truncated construct interacting with B56 $\delta$  indicating that the N-terminus is not essential for this interaction. B56 $\delta$  does not interact with  $\beta_{L125P}$  confirming that binding to  $\beta_4$  requires an intact SH3/GK interaction (Figure 4d). Endogenous B56 $\delta$  is expressed in both HEK293 and CHO cells

in agreement with spontaneous nuclear targeting of  $\beta_4$  (Supplementary Figure 3a,b). Exogenous B56 $\delta$ -myc co-localizes with nuclear  $\beta_4$ -EGFP and further increases  $\beta_4$ -EGFP nuclear targeting in CHO cells (Supplementary Figure 3c). Conversely, expression of a B56 $\delta$  shRNA, reducing the level of endogenous B56 $\delta$  in CHO cells (Supplementary Figure 3b), leads to a reduction of  $\beta_4$ -EGFP nuclear targeting (NCR value dropping from  $11.5 \pm 0.7$  to  $7.3 \pm 0.5$  or average 4.3-fold redistribution between nuclear and cytoplasmic pools by WB) (Figure 4e,f). In agreement with binding data, B56 $\delta$ -myc expression has no effect on  $\beta_{1-481}$ -EGFP distribution in CHO cells (Supplementary Figure 3d). Expression of B56 $\delta$ -myc in non-differentiated NG108-15 cells produces a marked nuclear re-localization of endogenous  $\beta_4$  (Figure 4g; 11.8-fold increase in NCR). The influence of B56 $\delta$  on endogenous  $\beta_4$  nuclear localization was further investigated using B56 $\delta^{-/-}$  mice (Louis et al, 2011). Hippocampal neurons from B56 $\delta^{-/-}$  mice show a significant 1.65-fold decrease in NCR value at 11 DIV (Figure 4h). Similarly, the density of immunogold-labeled endogenous  $\beta_4$  is significantly decreased by 1.61-fold in B56 $\delta^{-/-}$  adult hippocampal neurons compared to wt hippocampal neurons (Figure 4i). In conclusion, B56 $\delta$  is a SH3/GK conformation-sensitive  $\beta_4$  partner that contributes to nuclear distribution of  $\beta_4$ . Since a fraction of  $\beta_4$  is still nuclear in the absence B56 $\delta$ , we conclude that other B56 isoforms and/or nuclear partners also help  $\beta_4$  accumulation in neuronal nuclei.  $\beta_{1-481}$  deficient nuclear targeting originates from its inability to interact with B56 $\delta$ .

***$\beta_4$  forms a complex with B56 $\delta$  and active PP2A***

Immunoprecipitation of endogenous  $\beta_4$  from wt mice brain results in coprecipitation of B56 $\delta$  and PP2A (Figure 5a). As expected, neither B56 $\delta$  nor PP2A are immunoprecipitated from *lh* mice brain by  $\beta_4$  antibodies. Similarly, PP2A is not precipitated by  $\beta_4$  antibodies using B56 $\delta^{-/-}$  mice brain indicating that PP2A/ $\beta_4$  association requires B56 $\delta$ . In HEK293 cells, over-expression of B56 $\delta$ -EGFP with  $\beta_4$ -myc strongly enhances endogenous PP2A immunoprecipitation through  $\beta_4$ -myc (Figure 5b). Restricted immunoprecipitation of endogenous PP2A by antibodies against  $\beta_4$ -myc suggests that not all endogenous B56 $\delta$  (Supplementary Figure 3a) is associated to PP2A in these cells, possibly due to a competition with other B regulatory isoforms unable to associate to  $\beta_4$ -myc. As expected,  $\beta_{1-481}$ -myc mutant does not precipitate endogenous PP2A in the presence of B56 $\delta$ -EGFP (Figure 5b).  $\beta_4$ -myc/B56 $\delta$ -EGFP complex immunoprecipitated from HEK293 cells shows PP2A phosphatase activity (Figure 5c).

### ***Membrane depolarization triggers $\beta_4$ association to B56 $\delta$***

$\beta_4$ -EGFP, expressed together with Cacna1a and Cacna2d2, pore-forming and auxiliary subunits of VGCC, loses its nuclear distribution in HEK293 cells (Figure 5d, NCR =  $0.25 \pm 0.1$ , i.e. 40-fold reduction). Part of  $\beta_4$ -EGFP staining is cytoplasmic instead of at the plasma membrane, possibly by interaction with Cacna1a stuck in the endoplasmic reticulum. As expected,  $\beta_{1-481}$ -EGFP mutant distribution remains cytoplasmic in the presence of VGCC (Figure 5d, right panel). In the presence of Cacna1a and Cacna2d2, B56 $\delta$ -myc no longer interacts with  $\beta_4$ -EGFP (Figure 5e). These data indicate that (i) B56 $\delta$  does not form a higher order molecular complex with the channel and (ii)  $\beta_4$

association to Cacna1a overrides its interaction with B56δ. In addition, B56δ-EGFP does not affect VGCC kinetics and current densities in HEK293 cells (Supplementary Figure 4a,b). Also,  $\text{Ca}^{2+}$  currents were not modified in hippocampal neurons from B56δ<sup>-/-</sup> mice (Supplementary Figure 4c,d), confirming that B56δ does not associate to the channel complex. Previous reports evidenced that β/channel interaction is reversible (Restituito et al, 2001; Sandoz et al, 2004; Spafford et al, 2004), but the impact of membrane depolarization on this interaction has not been studied. Membrane depolarization of HEK293 cells enhances β<sub>4</sub>-myc interaction with B56δ-EGFP, while it does not promote any interaction between β<sub>1-481</sub>-myc and B56δ-EGFP (Figure 5f). In the presence of VGCC, the depolarization-induced B56δ-myc/β<sub>4</sub>-EGFP interaction requires external  $\text{Ca}^{2+}$  (Supplementary Figure 5). In these conditions, β<sub>4</sub>-myc precipitates endogenous PP2A only upon membrane depolarization (Figure 5g). In contrast, β<sub>1-481</sub>-myc does not precipitate PP2A upon depolarization. Finally, blocking electrical activity of 11 DIV hippocampal neurons by 1 μM tetrodotoxin (TTX) produces a significant 2.1-fold reduction of β<sub>4</sub> NCR value measured at 18 DIV (Figure 5h). This TTX effect is not observed in B56δ<sup>-/-</sup> hippocampal neurons, demonstrating that β<sub>4</sub> nuclear accumulation induced by electrical activity relies on B56δ. Note the 2.45-fold reduction in NCR value between wt and B56δ<sup>-/-</sup> control neurons at 18 DIV in agreement with B56δ role in β<sub>4</sub> nuclear accumulation. Conversely, a 1 hr stimulation of neuronal electric activity by a bicuculline/4-AP cocktail produces a significant  $23.5 \pm 1.9\%$  increase in NCR value that is not observed in B56δ<sup>-/-</sup> hippocampal neurons (Figure 5i). Depolarization of the plasma

membrane therefore represents an important signaling event that allows  $\beta_4$ /B56 $\delta$  interaction and favors  $\beta_4$  nuclear accumulation.

***$\beta_4$  represses TH gene expression by interacting with the thyroid hormone receptor alpha***

To determine whether  $\beta_4$  regulates transcription of endogenous genes, we analyzed a publicly available microarray data set comparing gene expression in wt and *lh* mice cerebellum (data set GSE6275 from Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo>). A volcano plot representation of our statistical analysis identifies 56 up-regulated and 38 down-regulated genes (over twofold expression change,  $p < 0.05$ ) in *lh* mice cerebellum (Supplementary Figure 6). Based on gene ontology annotations, clustering of regulated transcripts in functional groups illustrate that many encode proteins with functions related to signaling and ion transport (Supplementary Figure 6). Among these genes, those coding for TH and for BC031748 cDNA sequence are the most up- and down-regulated, respectively (Figure 6a). The effect of  $\beta_4$  on gene expression was confirmed on a set of genes by qRT-PCR experiments using purified mRNA from wt and *lh* mice cerebellum (Figure 6b). From both approaches, TH gene shows the greatest variation in expression level; an interesting observation considering its relevance in epilepsy (Bengzon et al, 1999; Donato et al, 2006; Hess & Wilson, 1991). The up-regulation of TH expression in *lh* mice cerebellum indicates that  $\beta_4$  acts as a repressor on this gene. Transcripts up- or down-regulation is lost in the hippocampus and in the cortex indicating the existence of compensatory mechanisms to the lack of  $\beta_4$ . Interaction of  $\beta_4$  with the promoter region of TH gene was

investigated by chromatin immunoprecipitation (ChIP) experiments. ChIP of the TH gene promoter from hippocampal neurons in culture, using antibodies against  $\beta_4$ , shows specific precipitation at 18 DIV but not 1 DIV (Figure 6c). As a control, ChIP of the 3'-UTR region of TH gene were negative at 18 DIV. Similar results were obtained for ChIP experiments of the transmembrane protein 100 gene promoter (Supplementary Figure 7), suggesting that the TH promoter is not an isolated case. In wt mice brain,  $\beta_4$  antibodies immunoprecipitate  $1.89 \pm 0.42\%$  of TH promoter input, corresponding to a 6.3-fold enrichment over control (Figure 6d). In contrast, no specific TH promoter immunoprecipitation is observed using *lh* mice brain (Figure 6d).  $\beta_4$  antibodies also immunoprecipitate  $1.37 \pm 0.06\%$  of TH promoter from B56 $\delta^{-/-}$  mice, indicating that  $\beta_4$  association with the promoter does not necessarily require the B56 $\delta$  partner (3.3-fold enrichment; Supplementary Figure 8a). Yeast two hybrid screening, using  $\beta_4$  as bait, was used to identify a transcription factor that targets  $\beta_4$  to the TH gene. The thyroid hormone receptor alpha (TR $\alpha$ ) came out as a positive candidate (Figure 6e). TR $\alpha$  is a nuclear receptor that contains a T3 hormone binding domain and a DNA binding domain interacting with Thyroid Receptor Elements (TRE). Interaction of TR $\alpha$  with  $\beta_4$  was confirmed by coimmunoprecipitation experiments in HEK293 cells (Figure 6f). However, this interaction is barely detectable in wt brain lysates probably because it constitutes a minor interacting fraction of total brain  $\beta_4$  (not shown). ChIP using TR $\alpha$  antibodies show that TR $\alpha$  can interact with the TH gene promoter regardless of the presence of  $\beta_4$  or B56 $\delta$  (Figure 6g). Enrichments of the TH gene promoter were 4.92-, 4.77- and 4.27-fold for wt, *lh* and B56 $\delta$  KO brains, respectively. This result is in agreement with the presence of putative TRE in the promoter region and in the coding

sequence of the TH gene. The effect of TR $\alpha$  on TH gene promoter was tested using a luciferase reporter (Figure 6h). TR $\alpha$  has little effect on its own on the expression of luciferase, while its activation by the T3 hormone leads to a 4.4-fold repression of luciferase expression (Figure 6h). Interestingly,  $\beta_4$ , like T3, activates the repressing function of TR $\alpha$  (4.8-fold repression). The effect of  $\beta_4$  on TR $\alpha$  activity may therefore constitute a first step of TH gene repression in agreement with the up-regulation of this gene in the cerebellum in the absence of  $\beta_4$ .

***B56 $\delta$  induces the recruitment of HP1 $\gamma$  by  $\beta_4$  and contributes to association of the signaling complex with histones***

ChIP experiments using B56 $\delta$  antibodies reveal the presence of B56 $\delta$  within the  $\beta_4$  complex associated to the promoter of the TH gene (Figure 7a). Control experiments using B56 $\delta$  KO mice brain demonstrate the specificity of the signal, while the absence of TH precipitation in *lh* brain denotes a key role of  $\beta_4$  in targeting B56 $\delta$  to the TH promoter. The presence of PP2A in the complex associated to the TH promoter was also confirmed by using PP2A antibodies (Figure 7b). TH promoter precipitation by PP2A antibodies was diminished in *lh* brain indicating the importance of  $\beta_4$  in recruiting PP2A to the TH gene (Supplementary Figure 8b). Next, we questioned the role of B56 $\delta$  and PP2A in TH gene regulation. Phosphorylation of histones regulates chromatin state and gene transcription (Jenuwein & Allis, 2001). Within the nucleus of wt CA1 hippocampal neurons, 45% of  $\beta_4$  gold-labeled is associated to heterochromatin (black arrows; n=195/440, Figure 1d), whereas the remaining fraction is associated to euchromatin. Expression of B56 $\delta$ -EGFP together with  $\beta_4$ -myc in HEK293 cells leads to a genome-

wide dephosphorylation of Ser<sup>10</sup> of histone H3 (Figure 7c), in agreement with earlier findings that PP2A dephosphorylates histone H3 (Nowak et al, 2003). This effect cannot be attributed to an increased population of transfected cells in G2/M phases of the cell cycle (Supplementary Figure 8c). In addition, immunoprecipitated  $\beta_4$ -myc/B56 $\delta$ -EGFP/PP2A-HA complex allows dephosphorylation of a Ser<sup>10</sup> phosphorylated histone H3 N-terminal peptide (Figure 7d). Finally, ChIP experiments using anti-histone H3 and anti-phosphorylated H3 Ser<sup>10</sup> antibodies shows that the absence of  $\beta_4$  in *lh* brain is accompanied by an increase of histone H3 Ser<sup>10</sup> phosphorylation at the TH promoter (Figure 7e). Ser<sup>10</sup> dephosphorylation of histone H3 has been associated to recruitment of HP1 $\gamma$  (Fischle et al, 2005). Interestingly, HP1 $\gamma$  has been shown to interact with a PXVXV motif on  $\beta_{4c}$  (Xu et al, 2011), a short splice variant of  $\beta_4$ . Although this motif is also found in full length  $\beta_4$  (Figure 7f), no interaction with HP1 $\gamma$  has been detected by yeast two hybrid (Figure 4c and ref. (Hibino et al, 2003)). Nevertheless, we questioned whether the interaction of  $\beta_4$  with B56 $\delta$  could control its interaction with HP1 $\gamma$ . Indeed, expression of B56 $\delta$ -EGFP strongly enhances immunoprecipitation of HP1 $\gamma$ -EGFP by  $\beta_4$ -myc (Figure 7g). The basal level of precipitation of HP1 $\gamma$ -EGFP by  $\beta_4$ -myc is due to the presence of endogenous B56 $\delta$  in HEK293 cells (Supplementary Figure 3a). The B56 $\delta$ -dependence of HP1 $\gamma$ / $\beta_4$  interaction, together with the PP2A-induced dephosphorylation of histone H3 Ser<sup>10</sup>, prompted us to investigate the interaction of this complex with histones. Immunoprecipitation of mice brain  $\beta_4$  leads to the precipitation of histone H3 (Figure 7h). The specificity of this association is witnessed by the lack of histone H3 precipitation from *lh* brain. Importantly,  $\beta_4$  association with histone H3 is lost in B56 $\delta^{-/-}$  mice brain, in agreement with the specific role of B56 $\delta$  in  $\beta_4$ /HP1 $\gamma$  interaction. In



HEK293 cells, immunoprecipitation of  $\beta_4$ -myc leads to the co-precipitation of all histones (H2B, H3 and H4) (Figure 7i), constitutive of nucleosomes. This is in agreement with the endogenous expression of B56 $\delta$  (Supplementary Figure 3a) and HP1 $\gamma$  (Supplementary Figure 3d) in these cells. Expression of B56 $\delta$  further enhances  $\beta_4$ -myc/histone interaction (Figure 7i). Similarly, B56 $\delta$ -myc requires the expression of  $\beta_4$ -EGFP to precipitate histone H3, in agreement with the fact that HP1 $\gamma$  binds  $\beta_4$  and not B56 $\delta$  (Figure 7j). B56 $\delta$ -myc expressed together with  $\beta_{L125P}$ -EGFP or  $\beta_{1-481}$ -EGFP mutants, deficient for B56 $\delta$  association, no longer precipitate histone H4 further supporting the B56 $\delta$ -dependence of  $\beta_4$ /HP1 $\gamma$  interaction (Figure 7k). Neuronal differentiation strongly increases the association of endogenous  $\beta_4$  with histone H3 in NG108-15 (Supplementary Figure 9a), which correlates with the differentiation-dependent  $\beta_4$  nuclear targeting (Figure 1h,i). In the presence of B56 $\delta$ -EGFP and channel subunits, membrane depolarization increases  $\beta_4$ -myc, but not  $\beta_{1-481}$ -myc, association to histone H3 (Supplementary Figure 9b). Coherent with its role as PP2A regulatory subunit and its genome-wide effect observed in Figure 7c, B56 $\delta$ -EGFP expression in HEK293 cells results also in dephosphorylation of Ser<sup>10</sup> of  $\beta_4$ -myc-associated histone H3 (Supplementary Figure 9c). ChIPs experiments using anti-HP1 $\gamma$  antibodies demonstrate the association of HP1 $\gamma$  to the TH promoter (Figure 7l). The strong decrease of this association in *lh* brain illustrates the role of  $\beta_4$  in recruiting HP1 $\gamma$  to the TH gene. Finally, double mutation of the HP1 $\gamma$  binding motif of  $\beta_4$  precludes the B56 $\delta$ -dependent interaction of  $\beta_4$  with histone H3 demonstrating that HP1 $\gamma$  or HP1 $\gamma$ -like proteins are required for  $\beta_4$  association to histones (Figure 7m).

### ***$\beta_4$ -dependent TH gene repression is mediated by electrical activity***

Our results indicate that in neurons there should be a  $\beta_4$ -dependent coupling between electrical activity and TH gene repression. To validate this conclusion, the electrical activity-dependence of TH mRNA levels was investigated using cerebellar granule neurons from wt and *lh* mice. Because these cells required depolarizing conditions to survive (25 mM KCl), which promotes basal level electrical activity, our control condition was defined as TH mRNA level after 2 days of TTX treatment (from 8 DIV to 10 DIV). Promoting electrical activity for one day, by removing TTX from 9 DIV to 10 DIV, induces strong TH mRNA level repression in wt (50%) but not in *lh* neurons (Figure 8a).

## **Discussion**

Here, we characterized a novel signaling pathway in which, under membrane depolarization, the VGCC  $\beta_4$  subunit accumulates in the nucleus and acquires a gene regulatory function by successively recruiting the B56 $\delta$ /PP2A complex, a transcription factor and the heterochromatin protein HP1 $\gamma$ . Nuclear localization of  $\beta_4$  was previously shown in mice brain by Subramanyam and coll. (Subramanyam et al, 2009). In contrast to what we observe with endogenous  $\beta_4$ , these authors show that depolarization of cultured neurons expressing V5-tagged  $\beta_4$  promotes nuclear export of the tagged protein. Besides the possibility that the V5 tag may interfere with the  $\beta_4$  / B56 $\delta$  interaction we describe and thus modify  $\beta_4$  nuclear targeting pathways, these results may highlight an active equilibrium between different pathways of  $\beta_4$  nuclear import and export. This

group identified the N-terminus of  $\beta_4$  as a possible determinant of nuclear accumulation. In support with the previous hypothesis, our own results show that this domain only partially interferes with  $\beta_4$  nuclear accumulation suggesting the existence of different pathways controlling  $\beta_4$  subcellular localization involving different  $\beta_4$  protein partners. We demonstrate here that the N-terminus of  $\beta_4$  is not important for its interaction with B56 $\delta$  suggesting that its contribution to nuclear accumulation occurs through a B56 $\delta$ -independent pathway. In our work, we focused on the  $\beta_4$  nuclear pathway that involves B56 $\delta$  and that is disrupted by the human epileptic mutation of  $\beta_4$ . We show that *in vitro* TR $\alpha$  acts as a transcription factor capable to interact with  $\beta_4$  and repress TH gene expression. The *in vivo* implication of TR $\alpha$  in controlling TH gene expression remains to be deciphered. The resulting nuclear platform, structured by  $\beta_4$ , binds to specific gene promoters, dephosphorylates histones associated to these promoters and orients HP1 $\gamma$  interaction with the dephosphorylated histones. The formation of this complex allows gene repression, first by a direct effect of  $\beta_4$  on TR $\alpha$ , and secondly by HP1 $\gamma$  recruitment known to promote heterochromatin formation (Figure 8b). This later step may lead to long-term gene repression, while the first step is conceptually more dynamic. In *lh* brain, a complete disorganization of this signaling pathway is observed in the absence of  $\beta_4$ . The combined reduction in HP1 $\gamma$  levels and greater phosphorylation level of histone H3 Ser<sup>10</sup>, are two events that conceptually may favor the up-regulation of TH expression. In that respect, further studies on the ability of nuclear  $\beta_4$  complex to induce other histone post-translational modifications, such as methylation or acetylation, will complete the characterization of the role of this complex in gene regulation. While our data demonstrate the key role of  $\beta_4$  in organizing TH gene repression, TH expression is not

uniformly up-regulated in all *lh* brain areas indicating the existence of compensatory mechanisms. In that respect, B56 $\gamma$ , that also targets PP2A to the nucleus (McCright et al, 1996), and  $\beta_3$ , another VGCC subunit, that is also addressed to the nucleus (Beguin et al, 2006) and binds B56 $\delta$  (Figure 4c) and TR $\alpha$  (unpublished observation), might participate to this compensation process. The juvenile epilepsy mutant that normally associates with VGCC (Escayg et al, 2000), is defective for B56 $\delta$  association. This mutant no longer forms a complex with PP2A, and is unable to translocate to the nucleus and to interact with HP1 $\gamma$  and histones (Figure 8b). Therefore, this mutation induces a full destabilization of the  $\beta_4$  signaling pathway. At the structural level, formation of the B56 $\delta$ / $\beta_4$  complex requires the interaction between the  $\beta_4$  SH3 and GK modules. The nuclear targeting defect induced by the human mutation resides in the loss of this interaction highlighting an unexpected role of  $\beta_4$  C-terminal domain in controlling  $\beta_4$  conformation state. Association of B56 $\delta$  with  $\beta_4$  promotes  $\beta_4$ /HP1 $\gamma$  interaction further indicating the exquisite modularity of  $\beta_4$  as an adaptor protein.

In earlier reports, B56 $\delta$ /PP2A was shown to regulate gene expression through a complex interplay between DARPP-32 phosphorylation state (Ahn et al, 2007), phosphatase 1 (PP1) activity, and histone H3 phosphorylation level (Stipanovich et al, 2008). PP1 complexes have been associated to other histone-modifying enzymes, such as histone deacetylases or kinases, with chromatin remodeling capabilities (Hsu et al, 2000; Murnion et al, 2001) and with transcription repressors or activators (Canettieri et al, 2003; Jin et al, 2003). Here, we illustrate that, like PP1, PP2A belongs to a signaling macromolecular complex which ultimately regulates gene expression by modulating the histone code. This complex is organized by the VGCC  $\beta_4$  subunit that plays the role of a

platform that, besides PP2A, also recruits a nuclear receptor and a heterochromatin protein, HP1 $\gamma$ . By homology with PP1, this protein complex is likely to be enriched by other nucleosome-modifying enzymes in the near future. It should be emphasized that long-term modifications accompanying epileptic disorders have previously been associated to changes in post-translational state of histones and to alterations in transcriptional programs (Huang et al, 2002; Sng et al, 2006). This work thus opens exciting perspectives for understanding the underlying molecular causes of epilepsy in which VGCC subunits have been implicated.

## **Material and Methods**

### ***Mice lines***

Lethargic (B6EiC3H-a/A-*lh*) and wild-type mice (strain B6EiC3H) were obtained from Jackson Labs and genotyped as previously described (Burgess et al, 1997). B56 $\delta^{-/-}$  mice were generated by exchanging 146 bp of exon 3, exons 4 to 14, and 45 bp of exon 15 of the *Ppp2r5d* gene with a neomycine cassette through homologous recombination (Louis et al, 2011). Animal procedures were run with the approval of the local ethical committee and all efforts were made to minimize both the suffering and number of animal used. Also, the experiments were carried out in accordance with the International Ethical Committee Guidelines (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996) for the care and use of laboratory animals.

### ***Electron microscopy***

Mice were fixed by intracardiac perfusion during 10 minutes with 50 ml of fixation buffer (2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.3). The hippocampus was then isolated and kept in fixation buffer during 2 days. A transversal slice was made in the mid third of the hippocampus and dissection of the CA1/CA3 area in this slice was performed under a binocular magnifier. Hippocampal neurons were cultured in B100 plates during 5 or 18 days and fixed 2 hrs with fixation buffer. Cells were gently detached with a cell scraper, centrifuged and embedded in 10% gelatine. The cell pellet was then cut in small pieces. 1 mm<sup>3</sup> cell or tissue samples were then incubated during 4 hrs in 2.3 M sucrose and frozen in liquid nitrogen. Cryosections were made at -120°C using a cryo-ultramicrotome (Leica-Reichert) and retrieved with a 1:1 solution of 2.3 M sucrose and 2% methylcellulose (Liou et al, 1996). Cryosections were first incubated with primary anti- $\beta_4$  (Everest Biotech, 1:100), then incubated with rabbit anti-goat antibodies (Jackson, 1:400) and revealed with protein A-gold conjugated (CMC, Utrecht). Labelled cryosections were viewed at 80 kV with a 1200EX JEOL TEM microscope and images were acquired with a digital camera (Veleta, SIS, Olympus). Gold particles were counted and surface area measured with iTEM software (analySIS).

### ***Hippocampal neurons, NG108-15 cell cultures and cerebellar granule neurons***

Primary cultures of hippocampal neurons were prepared as previously described (Peris et al, 2009). Astrocyte proliferation was blocked by 10  $\mu$ M arabinoside cytosine (AraC) four days after plating. Predominance of neurons was witnessed by Tuj1 labeling and lack of GFAP staining. NG108.15 cells were differentiated two days after plating by decreasing fetal calf serum to 1% and addition of 1 mM dibutyryl cAMP. Cell lines were

transfected using jetPEI and neurons at 9 DIV using calcium phosphate. Dissociated cerebellar granule neurons were prepared as previously described (Kiyonaka et al, 2007) except that 0.02% (w/v) trypsin (Difco) was used for dissociation of cerebellar tissues. Three hrs after cell plating, culture medium was replaced with Neurobasal (Invitrogen) with 2% B-27 (GIBCO), 26 mM KCl, 60 U/ml penicillin, and 60 µg/ml streptomycin, and renewed every 3 days. At 8 DIV, 0.1 µM TTX was added in the culture medium to block electrical activity. At 9 DIV, the TTX was washed out to promote electrical activity or maintained for continued electrical activity block. At 10 DIV, total RNA was isolated using NucleoSpin RNA XS (Macherey-Nagel) according to the manufacturer's instructions.

### ***Plasmid constructions***

cDNA constructs coding for EGFP-tagged  $\beta_4$  fragments were generated by PCR amplification using rat  $\beta_4$ -EGFP plasmid as template (Genbank accession code L02315) and subcloned into pEGFP-C1 using *HindIII* and *KpnI* sites. Myc-tagged  $\beta_4$  fragments were subcloned into pcDNA3.1(+) using *HindIII* and *XbaI* sites. For yeast two-hybrid assays, rat  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ,  $\beta_4$ , and  $\beta_4$  mutants were subcloned into pGBK-T7 vector (Kiyonaka et al, 2007). QuikChange® Site-Directed Mutagenesis Kit (Stratagene) or overlap extension method was used to produce  $\beta_{L125P}$ -EGFP,  $\beta_{P225R}$ -EGFP,  $\beta_{1-166-L125P}$ -EGFP,  $\beta_{P220A-V222A}$ -myc and  $\beta_{R28A-R29A-S30A}$ -EGFP. Mouse B56 $\delta$  (Genbank accession code NM009358), HP1 $\gamma$  (NM\_007624) and TR $\alpha$  (NM\_178060) were cloned from mouse brain Marathon-Ready cDNA (Clontech) using PCR and inserted into pCMV-tag3 vector and pEGFP-C2 vector using *EcoRI* and *SalI* site. The B56 $\delta$  and control shRNAs (sense

strand sequences: 5'-CATCGCATCTATGGCAAGTTT-3' and 5'-CAACAAGATGAAGAGCACCAA-3', respectively) were subcloned in TRC1.5 vector (Sigma). All constructs were sequenced. Human Cacna1a (Genbank accession code AF004883) and rat Cacna1a (code NM012919) were also used for expression experiments. The TH-luciferase reporter gene was as previously described (Robert et al, 1997) and contains 5,200 bps of the TH promoter upstream of the *Firefly* luciferase coding region.

### ***Cytochemical stainings***

Cells were fixed with 4% paraformaldehyde in PBS for 15 min. After PBS washing, plasma membranes were stained 5 min with 5 µg/ml concanavalin A-rhodamine. Cells were permeabilized with 0.1% Triton X-100. Myc-tagged constructs were visualized using biotinylated anti-myc antibodies (1:600, Santa Cruz) and Cy3-Streptavidin (1:300, Amersham). Endogenous  $\beta_4$  was labeled for 1-2 hrs with a goat antibody directed against its carboxyl-terminus (1/1,000 dilution, Everest Biotech), followed by Alexa488 conjugated anti-goat (1:500, Molecular Probes). Cell nuclei were visualized using ToPro3 dye (T3605; Molecular Probes Inc). Images were obtained with a confocal microscope (Leica SP2). NCR values correspond to the average measure of 50 cells. In each cell, fluorescence intensities were measured in forty different ROI (20 for the cytoplasm and 20 for the nucleus). Merged pictures were obtained by using Adobe Photoshop software.

### ***Yeast two hybrid assays***

Rat  $\beta_4$ , subcloned into pGBK-T7, was used as bait to screen a mouse brain pACT2 library expressed in the yeast strain AH109 (Clontech) (Kiyonaka et al, 2007).  $5.0 \times 10^6$



transformants were cultured with synthetic medium lacking adenine, histidine, leucine, and tryptophan. His<sup>+</sup> colonies were assayed in yeast cells for  $\beta$ -galactosidase activity by a filter assay. Signal strengths of interactions were evaluated visually after every 1 hr of color development. For mapping of the  $\beta_4$  region that interacts with B56 $\delta$  or HP1 $\gamma$ , yeast strain Y187 was co-transformed with each  $\beta$  bait vector and the B56 $\delta$  or HP1 $\gamma$  prey vector. The interactions were evaluated by  $\beta$ -galactosidase activity on a filter.

### ***Subcellular fractionation and Western blotting***

Transfected HEK293 cells were lysed with 10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5% Triton X-100 and protease inhibitor cocktail (Roche). Nuclei and cytoplasm were separated by 5 min centrifugation at 2,000 rpm. Pellets corresponding to nuclei were sonicated in lysis buffer. For mice brain subcellular fractionation, brains were homogenized in 0.32 M sucrose, 20 mM HEPES, pH 7.4, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche). The homogenate was centrifuged 10 min at 800 $\times$ g. The resulting crude nuclear pellet was washed twice with homogenization buffer, resuspended in a volume equivalent to the cytoplasmic fraction, and sonicated. Nuclear and cytoplasmic (supernatant) proteins, from equal volumes, were separated by SDS-PAGE and analyzed by Western blot. EGFP tagged-proteins were visualized using anti-EGFP antibodies (1:300, Santa Cruz or 1:5,000 Rockland) and anti- $\beta_4$  as described (Kiyonaka et al, 2007).

### ***Immunoprecipitation experiments***

Transfected cells were lysed 30 min in 10 mM Tris pH 7.4, 1.5 mM MgCl<sub>2</sub>, 60 mM KCl, 15 mM NaCl, 0.5% Triton X-100 supplemented with protease inhibitor cocktail (Roche), sonicated and centrifuged 5 min at 1,500×g. Similar procedure applies for mice brain, except that they were homogenized first in lysis buffer. Protein extracts were incubated with streptavidin beads (Dynabeads® Streptavidin M-280, Dynal) precoated with 3 µg of biotinylated anti-myc antibody (Santa Cruz Biotechnology Inc.). For immunoprecipitation of endogenous β<sub>4</sub>, protein extracts were incubated with 40 µl of dynabeads® protein G (Dynal) precoated with anti-β<sub>4</sub> antibodies (Everest Biotech). Beads were washed with PBS/0.1% Tween 20. Immunoprecipitated proteins were eluted with denaturing buffer and analyzed by Western blot. For immunoprecipitation of phosphorylated histones, lysis buffer was supplemented with 50 mM NaF phosphatase inhibitor. For immunoprecipitation and ChIP, primary antibodies were used at 10 µg: anti-β<sub>4</sub> from Everest or as described (Kiyonaka et al, 2007), anti-histone H2B from Upstate, anti-histone H3, anti-phosphorylated Ser<sup>10</sup> histone H3, anti-histone H4, anti-HP1γ, and anti-TRα from Abcam, anti-B56δ as described in (Martens et al, 2004), anti-PP2A from Bethyl laboratories Inc., and anti-actin from Sigma-Aldrich.

### ***PP2A activity***

PP2A activity of immunoprecipitates was measured using SensoLyte™*p*NPP protein phosphatase kit from AnaSpec. Incubation lasted 1 hr at 37°C in assay buffer in the presence of *p*-nitrophenyl phosphate (pNPP). OD was measured at 405 nm.

### ***Quantitative RT-PCR***

Total RNAs were extracted from wt, *lh* or B56 $\delta^{-/-}$  cerebellum using the RNeasy kit, supplemented with DNase I (Qiagen). cDNAs were synthesized from 1  $\mu$ g total RNA using random hexamer primers (Promega) and Superscript II (Invitrogen). Primers recognizing mice sequences were designed with the Primer 3 software (<http://frodo.wi.mit.edu/>) or by Sigma service (Supplementary Figure 10). Real time PCR was performed with a light cycler 480 instrument using the Lightcycler LC480 SYBR green I master (Roche) according to the manufacturer's instructions. The cycling protocol was 10 min at 95°C followed by 40 cycles of 3 steps each (10 sec at 95°C, 5 sec at 60°C, and 10 sec at 72°C). The specificity of the amplification was checked by generating a melting curve ranging from 65 to 95°C. Multiple normalization of gene expression (Vandesompele et al, 2002) was performed using 3 housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, transferrin receptor, and peptidylprolyl isomerase A). For TH expression levels in cerebellar granule neurons, cDNAs were synthesized using AMV reverse transcriptase XL and an oligo dT-adaptor primer (TaKaRa). Real time PCR and cycling protocol were performed as above, except for the number of cycles (50). TH gene expression was normalized relative to rpl27 (de Jonge et al, 2007). Values were expressed relative to wt treated with TTX, which was given the arbitrary value of 1.

### ***Chromatin immunoprecipitation***

Frozen adult mice brain (wt, *lh* or B56 $\delta^{-/-}$ ) brain were first crunched into powder in a mortar filled with liquid nitrogen. This powder was resuspended in 1% formaldehyde (in PBS) and incubated for 20 min at RT. Cross linking was stopped by addition of glycine (125 mM final concentration) and incubation 5 min. Fixed tissues were thus washed with

i) cold PBS (5 min), ii) 10 mM HEPES, 0.5 mM EGTA, 10 mM EDTA and Triton X-100 0.25%, pH 7.4 (10 min), and iii) 10 mM HEPES, 0.5 mM EGTA, 1 mM EDTA and 200 mM NaCl, pH 7.4 (10 min). Samples were then solubilized in 1 ml of buffer containing 50 mM Tris, pH 7.4, 10 mM EDTA, 1% SDS for 2 hrs at 4°C, sonicated until reaching an average 500-1,000 bps DNA fragment size, and centrifuged 5 min at 20,000 g. 100 µl of lysate ( $DO_{280} = 0.266$ ) were incubated overnight at 4°C in 1 ml of 0.01% SDS, 1.1% Triton X100, 1.2 mM EDTA, 16 mM Tris pH 8.0 and 170 mM NaCl, 1 mM PMSF, and protease inhibitor cocktail, with or without 10 µg antibodies. Immunoprecipitation was performed by adding dynabeads protein G (Invitrogen). Different washing steps and elution were performed as described (Dahl & Collas, 2008). Cross-links were reversed overnight at 65°C. DNA samples were sequentially treated with RNase A and proteinase K. DNA was extracted by phenol/chloroform and ethanol precipitated with glycogen as a carrier. DNA pellet was dissolved in water and real-time quantitative PCR analyses were performed on 25 ng of DNA in duplicate with selected primers (Supplementary Figure 10). On cultured hippocampal neurons, ChIP experiments were conducted on  $5 \times 10^6$  cells using the Magna ChIP<sup>TM</sup> (MILLIPORE) according to the manufacture's protocol. The PCR cycling protocol was 10 min at 94°C followed by 38 cycles of 3 steps each (30 sec at 94°C, 30 sec at 55°C or 61°C, and 30 sec at 72°C), and 7 min at 72°C. Annealing temperature for the promoter region of TH was 55°C and for the 3'UTR of TH was 61°C.

### ***Mass spectrometry***

The histone H3 peptide phosphorylated at Ser<sup>10</sup> QTARKS<sub>p</sub>TGGC was obtained from Smartox biotechnologies (Grenoble, France). Immunoprecipitates were washed with (in

mM) 40 Tris pH 8.4, 34 MgCl<sub>2</sub>, 4 EDTA, 4 DTT, and incubated overnight at 37°C with 1 mM peptide. Dephosphorylation was evaluated by MALDI-TOF (Applied 4800) mass spectrometry. Briefly, 1 µl of peptide solution was spotted directly onto the MALDI target, dried and 1 µl of 20% α-Cyano-4-hydroxy-cinnamic acid (Sigma) was added in 50% acetonitril, 0.1% trifluoroacetic acid.

## **Acknowledgments**

We are indebted to Dr. Hiroshi Hibino (Osaka University, Japan) for providing HP1γ constructs used for coimmunoprecipitation, confocal imaging and yeast two hybrid assays. We thank Dr. Rosalie Sears (Oregon Health & Sciences University, Portland, USA) for the pD30-PP2A-HA-C clone. M.B. is a postdoctoral fellow of Association Française contre les Myopathies. The authors declare that they have no conflict of interest.

*Author contributions:* A.T. and K.F. studied the nuclear localization of β<sub>4</sub> and its structural determinants. Sh.K., T.M., S.A. made several cDNA constructs and performed yeast two-hybrid and ChIP experiments. M.B., K.F. and Mi.R. performed RT-PCR, immunoprecipitation and ChIP experiments. C.A. did electrophysiological recordings. S.G-F. and A.A. provided their expertise with hippocampal neuron cultures. K.S. performed mass spectrometry experiments and Sa.K. contributed to discussion, design and interpretation of data regarding nuclear function of β<sub>4</sub> complex. F.L. performed CHCB2 experiments. K.P-G. did EM experiments. S.B. performed transcriptomic analyses. C.L. and V.J. provided the B56δ<sup>-/-</sup> mice line and analytical tools. R.D., Mi.R., Y.M. and M.D.W. coordinated the study. Mi.R. and M.D.W. wrote the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Figure legends

**Figure 1** Presence of  $\beta_4$  in the nucleus of neurons. **(a)** Immunohistochemical confocal images of 20  $\mu\text{m}$  coronal sections of adult wt (upper panels) or *lh* (lower panels) mice brains showing the distribution of endogenous  $\beta_4$  in the dentate gyrus (green, left panels). Neuronal nuclei were labeled with NeuN (blue, middle panels). Right panels illustrate merged images. The inserts show 5X images of the CA3 region (initially 20X). **(b)** Western blots of adult wt and *lh* mice brain lysates indicating the presence of  $\beta_4$  only in wt mice. Actin staining was used as an internal control. **(c)**  $\beta_4$  immunoblotting from cytoplasmic and nuclear fractions of adult wt mice brain.  $\beta$ -tubulin and nucleolin are used as indicators of nuclear and cytoplasmic fraction purities, respectively. **(d)** EM image of an ultrathin cryosection of the CA1 hippocampal region showing the intranuclear presence of  $\beta_4$  labelled with antibody-coated 15 nm gold particles. Black and white arrows indicate the position of gold particles associated to heterochromatin and euchromatin, respectively. **(e)** Confocal images of hippocampal neurons from embryonic E18 mice brains in primary culture at 5 DIV (left panels) and 18 DIV (right panels). Green: immunocytochemical staining of  $\beta_4$ ; red: membrane staining with concanavalin A (ConA) conjugated to rhodamine; blue: nuclear staining with ToPro3. **(f)** DIV-dependent evolution of  $\beta_4$  NCR values in neurons (n=50 cells for each data point). **(g)** Nuclear density of  $\beta_4$  in hippocampal neurons at 5 and 18 DIV as assessed by EM (n=28 and 20 nuclei, respectively). **(h)** Confocal images of NG108.15 cells 1 day before (-1 DIV) and 13 days (13 DIV) after induction of neuronal differentiation with 1 mM cAMP and serum

reduction. Color code is as in (e). Differentiated NG108-15 cells have larger nuclei than non-differentiated cells. (i) Mean NCR values expressed in percent as a function of culture time *in vitro*. DIV 0 represents the induction time of neuronal differentiation.

**Figure 2** Nuclear targeting and structural integrity of  $\beta_4$  are disrupted by the human juvenile epilepsy mutation. (a) Schematic representation of different truncated  $\beta_4$  constructs in pEGFP-C1. The  $\beta_{1-481}$ -EGFP mutant is also shown. (b) Confocal images of CHO cells expressing some representative constructs, including  $\beta_4$ -EGFP and mutant  $\beta_{1-481}$ -EGFP. Expression time is 24 hrs after transfection. (c) Mean NCR values of EGFP fluorescence for each condition in transfected CHO cells. \*\*\*,  $p \leq 0.01$ . (d) Western blot detecting EGFP in nuclear and cytoplasmic fractions of CHO cells after two days of transfection with EGFP,  $\beta_4$ -EGFP or  $\beta_{1-481}$ -EGFP. Cytoplasmic  $\beta_{1-481}$ -EGFP is less stable than cytoplasmic  $\beta_4$ -EGFP, as witnessed by the presence of lower molecular weight bands. These truncated constructs are deficient in nuclear accumulation as shown in (c), possibly because containing GK domains. (e) Confocal images of hippocampal neurones transfected at 7 DIV with  $\beta_{1-481}$ -EGFP (upper panels) or  $\beta_4$ -EGFP (lower panels). Images were acquired at 9 DIV on  $\beta$ -tubulin III-positive cells. (f) NCR values for each construct. \*\*\*,  $p \leq 0.01$ . (g) Confocal images of hippocampal neurons transfected with  $\beta_{1-481}$ -EGFP showing the comparative distribution of endogenous  $\beta_4$  (red) and exogenous  $\beta_{1-481}$ -EGFP (green).

**Figure 3** The SH3/GK interaction is required for  $\beta_4$  nuclear localization. (a) EGFP- or myc-tagged  $\beta_4$ -truncated constructs used for confocal microscopy and co-

immunoprecipitation experiments. **(b)** Confocal images of CHO cells transfected with individual constructs. **(c)** Confocal images of CHO cells expressing  $\beta_{1-166}$ -EGFP with  $\beta_{200-519}$ -myc, or  $\beta_{1-166}$ -EGFP with  $\beta_{200-481}$ -myc (upper panels). Mean NCR values of EGFP or anti-myc fluorescence summarizing the effect of the co-expressions on nuclear localization (lower panels). \*\*\*,  $p \leq 0.001$ . **(d)** Immunoprecipitation experiments in CHO cells investigating the association of  $\beta_{1-166}$ -EGFP with  $\beta_{200-519}$ -myc or  $\beta_{200-481}$ -myc. Left panel: immunoprecipitation via anti-myc antibodies and Western blot of EGFP, except first lane showing  $\beta_4$ -EGFP expression level in cell lysates. Right panel: Western blot with polyclonal anti- $\beta$  antibody (Bichet et al, 2000) indicating equivalent  $\beta_{200-519}$ -myc and  $\beta_{200-481}$ -myc immunoprecipitation levels.  $\beta_{1-166}$ -EGFP expression confirmed by cell EGFP fluorescence. **(e)** Schematic representation of  $\beta_{L125P}$ -EGFP,  $\beta_{1-166-L125P}$ -EGFP and  $\beta_{P225R}$ -EGFP mutants. **(f)** Lack of co-immunoprecipitation shows the absence of  $\beta_{200-519}$ -myc/ $\beta_{1-166-L125P}$ -EGFP interaction. Expression of  $\beta_{1-166}$ -EGFP and  $\beta_{200-519}$ -myc was verified by the cell fluorescence and Western blot as in **(d)**. **(g)** Confocal images of hippocampal neurons showing the cytoplasmic localization of  $\beta_{L125P}$ -EGFP and  $\beta_{P225R}$ -EGFP. Cells were transfected for 48 hrs at 6 DIV. **(h)** Mean NCR values for  $\beta_4$ -EGFP,  $\beta_{L125P}$ -EGFP and  $\beta_{P225R}$ -EGFP fluorescence in hippocampal neurons. \*\*\*,  $p \leq 0.001$ .

**Figure 4** B56 $\delta$ / $\beta_4$  interaction contributes to  $\beta_4$  nuclear localization. **(a)** Yeast two hybrid results indicating the interaction level of  $\beta_4$  and  $\beta_{1-481}$  with B56 $\delta$  as a function of time (left). The interactions were scored as the ratio of  $\beta$ -galactosidase activity to His prototrophy. Schematic representation of mouse B56 $\delta$  showing the NLS amino acid

sequence (right). The position of seven HEAT repeats are shown by homology with B56 $\gamma$  (Magnusdottir et al, 2009). **(b)** Co-immunoprecipitation experiments determining the interaction between  $\beta_4$ -EGFP or  $\beta_{1-481}$ -EGFP with B56 $\delta$ -myc in HEK293 cells. Left panel: expression of B56 $\delta$ -myc was confirmed by Western blot (Mr 69 kDa). Right panel: pull-down with anti-myc antibody, and Western blot with anti-EGFP antibody. **(c)** Interactions of  $\beta$  isoforms with B56 $\delta$  and HP1 $\gamma$  in yeast two hybrid assay. **(d)** Interactions of truncated and mutated  $\beta_4$  constructs with B56 $\delta$  using yeast two hybrid assay. **(e)** Effect of B56 $\delta$  shRNA and control shRNA on the NCR value of  $\beta_4$ -EGFP in CHO cells. \*\*\*,  $p \leq 0.001$ ; NS,  $p \geq 0.1$ . **(f)** Effect of B56 $\delta$  shRNA and control shRNA on the nuclear / cytoplasmic distribution of  $\beta_4$ -EGFP in CHO cells, as assessed by WB. Histone H3 and  $\beta$ -tubulin are used as markers of the nucleus (N) and cytoplasm (C), respectively. Performed in duplicate. Average ratios of  $4.2 \pm 0.32$  ( $\beta_4$ -EGFP) and  $0.97 \pm 0.81$  ( $\beta_4$ -EGFP + shRNA B56 $\delta$ ). **(g)** Confocal images showing the cell distribution of endogenous  $\beta_4$  in undifferentiated NG108.15 cells in the absence or presence of B56 $\delta$ -myc (left panels). B56 $\delta$ -myc was transfected 24 hrs before confocal imaging. Panels in green present the endogenous  $\beta_4$  detected with an anti- $\beta_4$ . Mean NCR values showing that B56 $\delta$ -myc induces the redistribution of endogenous  $\beta_4$  to the nucleus (right panel). \*\*\*,  $p \leq 0.001$ . **(h)** Mean NCR values of endogenous  $\beta_4$  from wt and B56 $\delta^{-/-}$  hippocampal neurons at 11 DIV. \*\*\*,  $p \leq 0.001$ . **(i)** Average immunogold-labeled endogenous  $\beta_4$  in nuclei from pyramidal neurons from wt and B56 $\delta^{-/-}$  mice hippocampus measured by EM (n=36 and 51 nuclei, respectively). \*\*\*,  $p \leq 0.001$ . Control counts in *lh* hippocampus were  $0.51 \pm 0.36$  gold particles/ $\mu\text{m}^2$ .

**Figure 5**  $\beta_4$  association to B56 $\delta$ /PP2A is inhibited by channel expression and activated by membrane depolarization. **(a)**  $\beta_4$  immunoprecipitates B56 $\delta$  and PP2A from wt mice brain but not from *lh* or B56 $\delta^{-/-}$  mice as shown by Western blots. **(b)** Left panel: immunoprecipitation of endogenous PP2A by B56 $\delta$ -myc or  $\beta_4$ -myc  $\pm$  B56 $\delta$ -EGFP expressed in HEK293 cells. Right panel: similar experiments using  $\beta_{1-481}$ -myc  $\pm$  B56 $\delta$ -EGFP. **(c)** Endogenous PP2A phosphatase activity associated to immunoprecipitated  $\beta_4$ -myc  $\pm$  B56 $\delta$ -EGFP expressed in HEK293 cells. Dephosphorylation of *p*-nitrophenyl phosphate (*p*NPP), a generic phosphatase substrate, was measured by absorbance of the metabolite at 405 nm. Experiment in duplicate. **(d)** Confocal images of HEK293 cells 2 days after transfection with  $\beta_4$ -EGFP together with Cacna1a and Cacna2d2 subunits (VGCC) (left panel). Mean NCR values of EGFP fluorescence in various transfection conditions (n=50 for each condition, right panel). **(e)** Western blot analysis of  $\beta_4$ -EGFP subunit immunoprecipitated by B56 $\delta$ -myc  $\pm$  VGCC expressed in HEK293 cells. Control IP represents precipitation in absence of anti-myc antibodies. **(f)** Western blot analysis of B56 $\delta$ -EGFP immunoprecipitated by  $\beta_4$ -myc (left panels) or  $\beta_{1-481}$ -myc (right panels) expressed in HEK293 cells together with VGCC, without or with a 30 min depolarization by 140 mM KCl. **(g)** Effect of HEK293 membrane depolarization (140 mM KCl, 30 min) on  $\beta_4$ -myc or  $\beta_{1-481}$ -myc complex formation with PP2A in the presence of B56 $\delta$ -EGFP and VGCC. **(h)** Mean NCR values of  $\beta_4$  immunofluorescence in wt or B56 $\delta^{-/-}$  hippocampal neurons at 18 DIV (control or 1  $\mu$ M TTX). TTX was added at 11 DIV in the culture media (n=50 for each condition). **(i)** Effect of a 1 hr application of 40  $\mu$ M

biccuculine and 400  $\mu$ M 4-AP on average  $\beta_4$  NCR value in wt and B56 $\delta^{-/-}$  hippocampal neurons. NS, non significant; \*\*\*,  $p < 0.001$ .

**Figure 6**  $\beta_4$  regulates gene expression by interacting with a transcription factor. **(a)** Histogram showing up-regulated (in red) and down-regulated (in green) genes displaying a  $> 2$ -fold change in *lh* versus wt cerebellum mRNA levels as detected with transcriptomic probe sets. **(b)** qRT-PCR experiments showing up-regulation of TH, TAC1, KLB and down-regulation of BC031748 and RIF1 in *lh* versus wt cerebellum. Mean  $\pm$  S.D. of 3 experiments performed in triplicate. Data were normalized using 3 housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, transferin receptor, and peptidylprolyl isomerase A). **(c)** Agarose gel of representative ChIP assay on hippocampal neurons at 1 or 18 DIV with anti- $\beta_4$  antibodies. **(d)** TH promoter immunoprecipitation in presence or absence (control) of anti- $\beta_4$  antibodies, expressed as percentage of input, from wt or *lh* mice brain (n=4-22, left panel). \*\*\*,  $p < 0.001$ . **(e)** Yeast two hybrid results indicating the interaction level of  $\beta_4$  with TR $\alpha$  as a function of time (upper panel). The interactions were scored as the ratio of  $\beta$ -galactosidase activity to His prototrophy. Schematic representation of mouse TR $\alpha$  showing the localization of the DNA Binding Domain (DBD) and the Ligand Binding Domain (LBD) (lower panel). **(f)** Western blot analysis of TR $\alpha$ -EGFP immunoprecipitated by  $\beta_4$ -myc expressed in HEK293 cells. **(g)** TH promoter immunoprecipitation by anti-TR $\alpha$  antibodies, expressed as percentage of input, from wt, *lh* or B56 $\delta^{-/-}$  mice brain (n=4-20, left panel). \*\*\*,  $p < 0.001$ . **(h)** Schematic representation of the luciferase reporter system under the control of TH promoter (upper panel). Luciferase expression measured in the absence and



presence of T3 in HEK293 cells transfected with the luciferase reporter alone or together with TR $\alpha$   $\pm$   $\beta_4$  (lower panel). An everted repeat type 6 TRE is present in the promoter region of TH gene (TGGCCTTGCCTGAGGCCA) at position -341 to -323.

**Figure 7** Association of  $\beta_4$  to HP1 $\gamma$  and histones requires B56 $\delta$ . **(a)** TH promoter immunoprecipitation by anti-B56 $\delta$  antibodies, expressed as percentage of input, from wt, *lh* or B56 $\delta^{-/-}$  mice brain (n=2-8). \*\*, p<0.05. **(b)** TH promoter immunoprecipitation by anti-PP2A antibodies, expressed as percentage of input, from wt mice brain (n=12). \*\*, p<0.05. **(c)** Western blot of H3 and H3 Ser<sup>10</sup>P in HEK293 cells expressing  $\beta_4$ -myc alone or with B56 $\delta$ -EGFP. **(d)** Mass spectrometry data showing dephosphorylation of the phosphorylated N-terminal histone H3 peptide (amino acids 5 to 13 phosphorylated on Ser<sup>10</sup>) by  $\beta_4$ -myc immunoprecipitated from HEK293 cells, cotransfected with B56 $\delta$ -EGFP and PP2A-HA. **(e)** TH promoter immunoprecipitation by anti-Ser<sup>10</sup>P H3 or anti-H3 antibodies, expressed as percentage of input, from wt or *lh* mice brain (n=3-22). \*, p<0.1. **(f)** Schematic representation of  $\beta_{4c}$  and  $\beta_4$  illustrating the localization of the HP1 $\gamma$  binding motif (critical residues are shadowed). **(g)** Western blot of HP1 $\gamma$ -EGFP following immunoprecipitation by anti-myc antibodies from HEK293 cells expressing various combinations of HP1 $\gamma$ -EGFP, B56 $\delta$ -EGFP and  $\beta_4$ -myc. **(h)** Immunoprecipitation of histone H3 from wt, *lh* or B56 $\delta^{-/-}$  brain by anti- $\beta_4$  antibodies. **(i)** Immunoprecipitation of H3, H2B and H4 histones from HEK293 cells by  $\beta_4$ -myc in the absence or presence of B56 $\delta$ -EGFP. Control: immunoprecipitation in non-transfected cells (NT). **(j)** Immunoprecipitation of histone H3 in HEK293 cells by B56 $\delta$ -myc in the absence or presence of  $\beta_4$ -EGFP. **(k)** Lack of co-immunoprecipitation of histone H4 and B56 $\delta$ -myc

in HEK293 cells in the presence of  $\beta_{L125P}$ -EGFP or  $\beta_{1-481}$ -EGFP mutants. **(l)** TH promoter immunoprecipitation by anti-HP1 $\gamma$  antibodies, expressed as percentage of input, from wt or *lh* mice brain (n=4-14). \*\*,  $p<0.05$ . **(m)** Mutation of the HP1 $\gamma$  binding motif prevents  $\beta_4$ -myc association to histone H3 in the presence of B56 $\delta$ -EGFP.

**Figure 8** Experimental validation and schematic illustration of the novel excitation-transcription pathway mediated by  $\beta_4$ . **(a)** Electrical activity reduces TH mRNA levels in primary cultures of cerebellar granule neuronal from wt mice but not *lh* mice. Upper panel represents the experimental protocol. First condition in which TTX is added for 2 days (8 DIV to 10 DIV) is used as control condition without electrical activity (blue colour), whereas second condition in which TTX is washed out at 9 DIV is used as the condition in which electrical activity is restored (red colour). Lower panel illustrates the fold-change in TH mRNA level induced by electrical activity using *rpl27* as housekeeping gene and normalisation to control condition. \*\*,  $p<0.05$ . **(b)** Schematic representation of the new signalling pathway and of the defective steps produced by the epileptic truncation of  $\beta_4$ .

Figure 1 (De Waard)

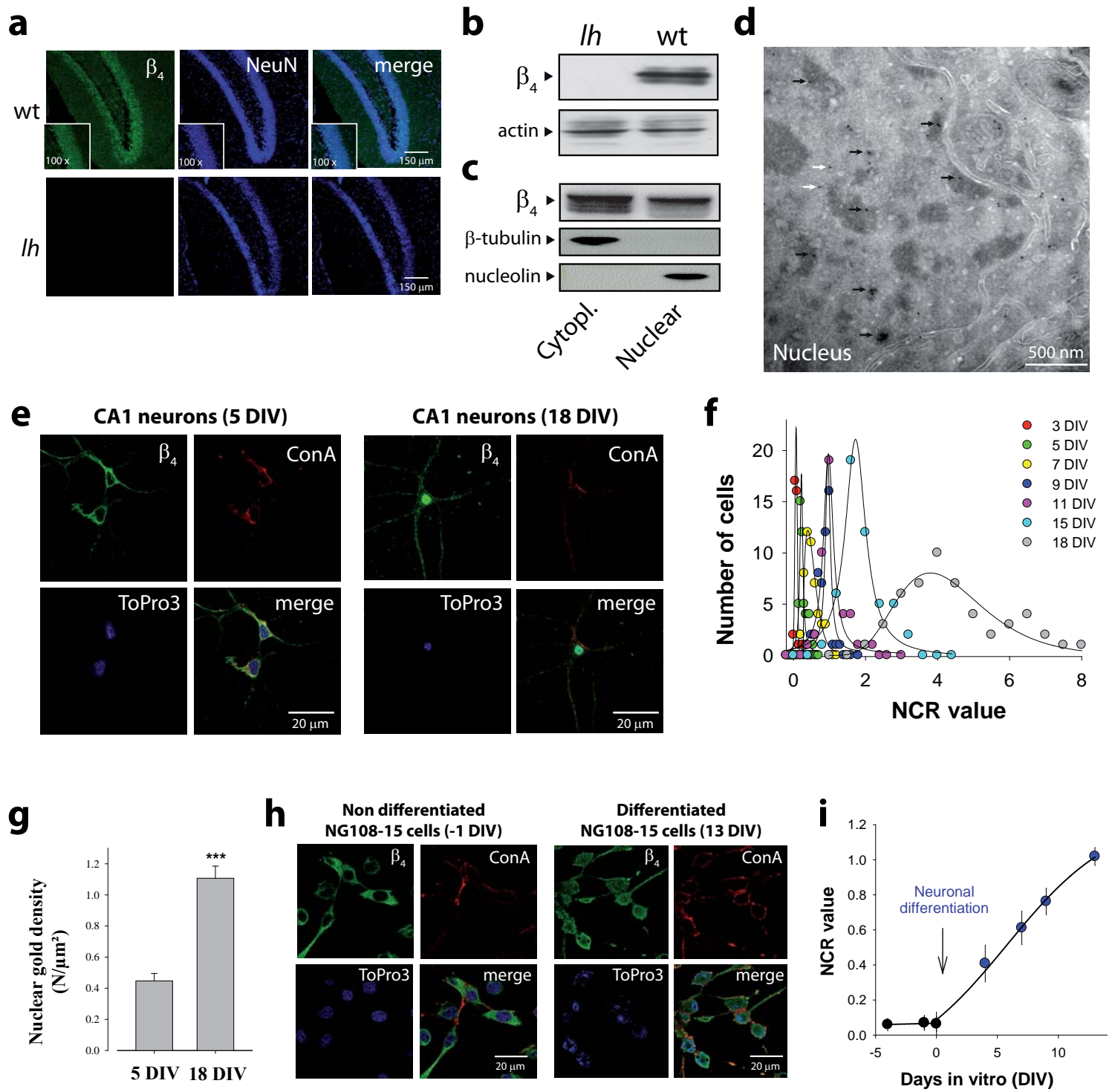


Figure 2 (De Waard)

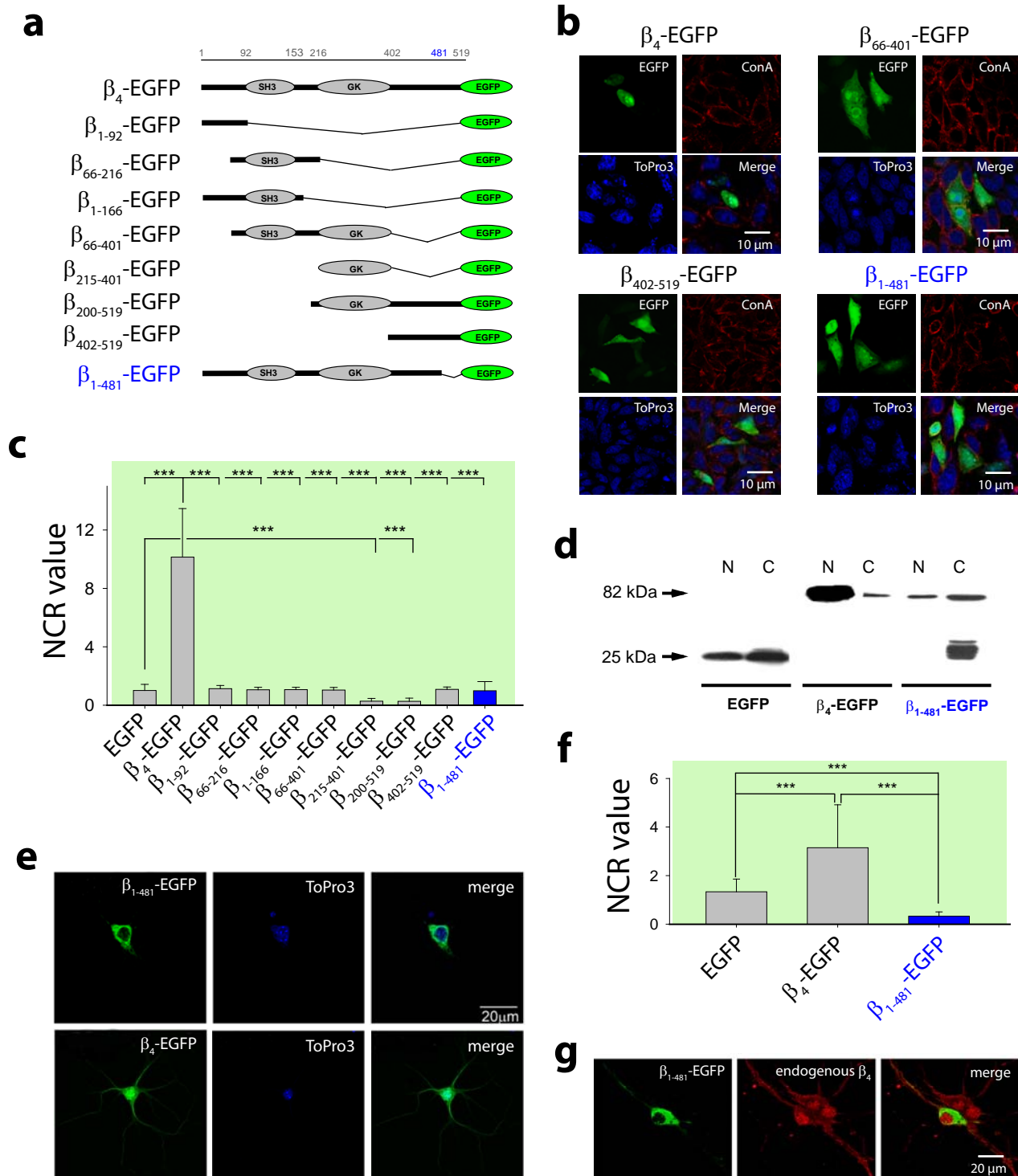


Figure 3 (De Waard)

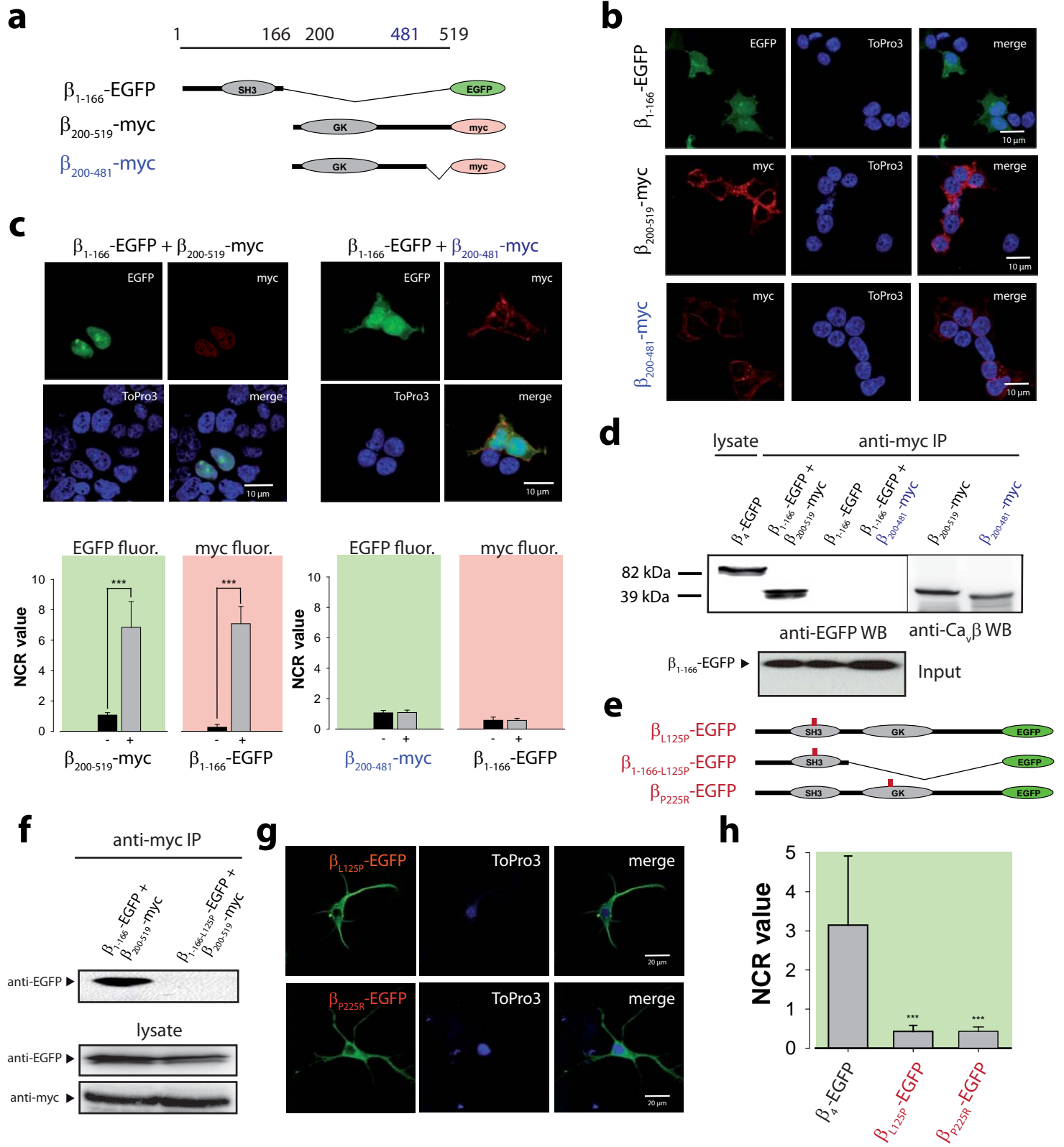
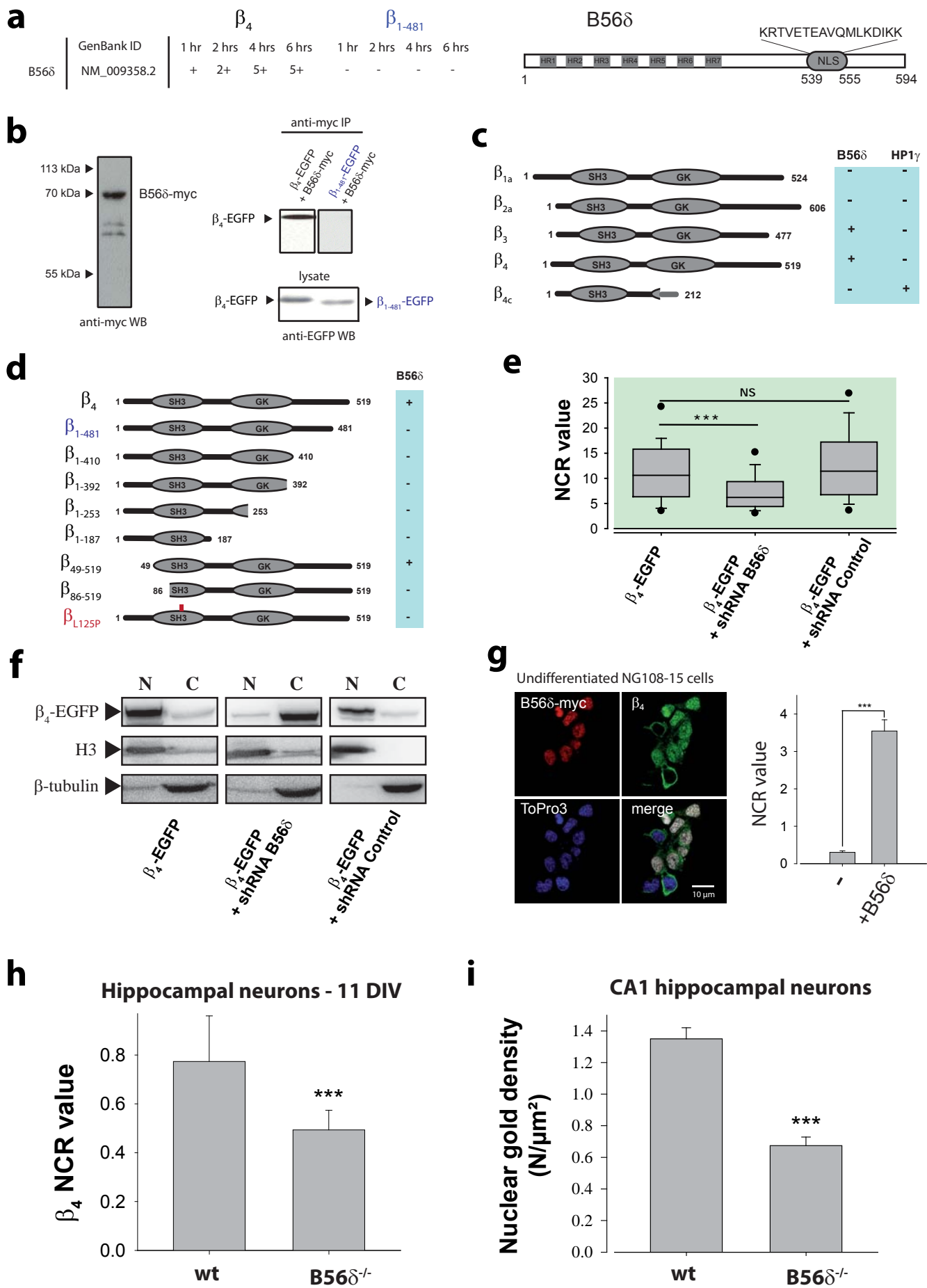


Figure 4 (De Waard)



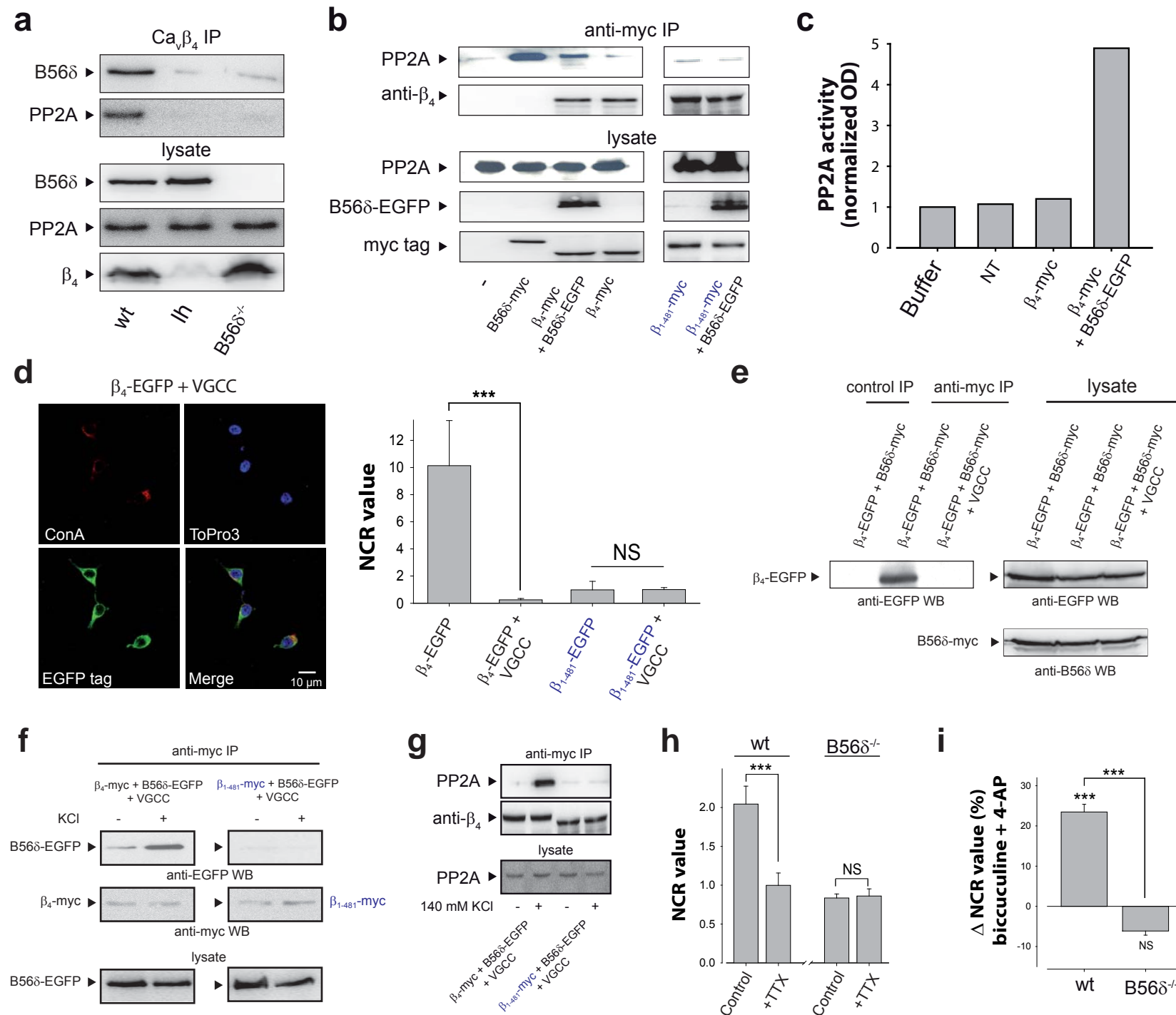


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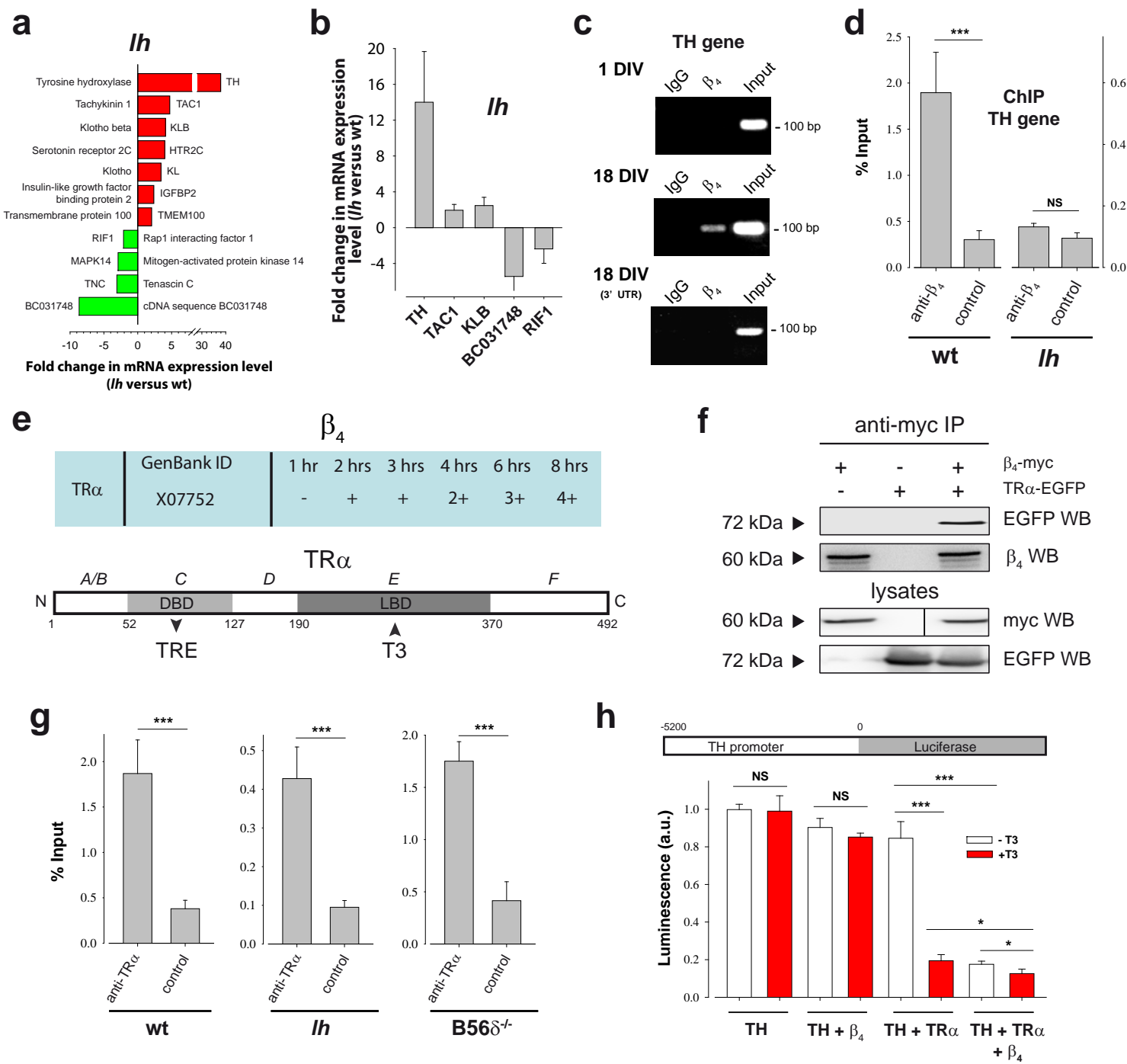




Figure 7 (De Waard)

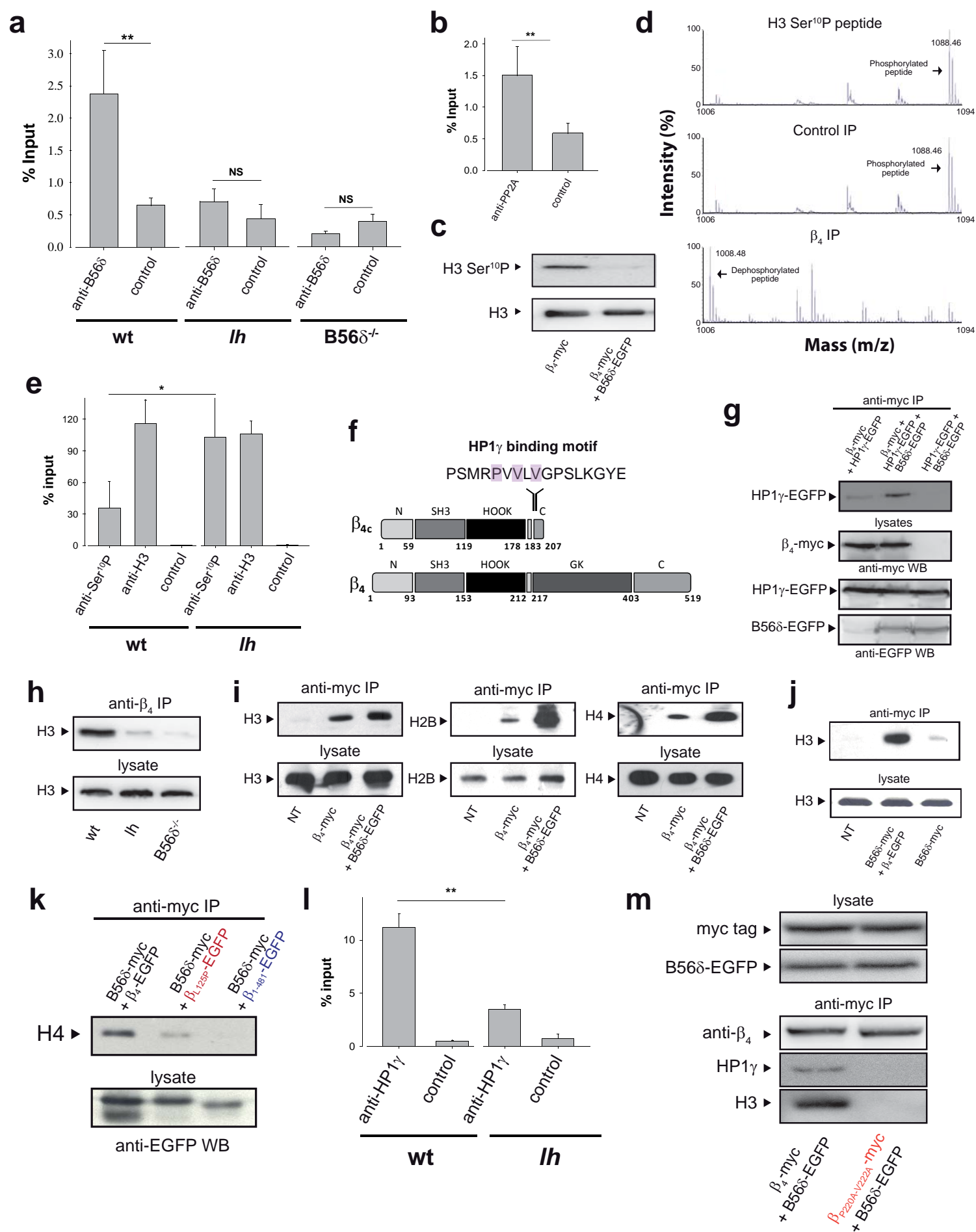
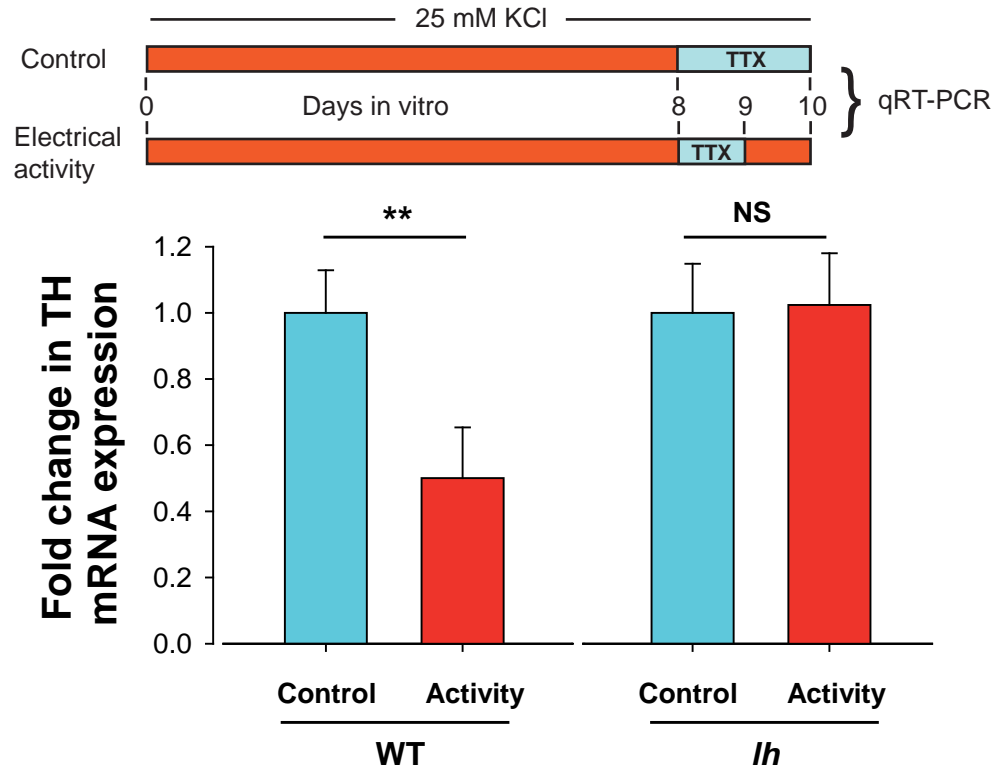


Figure 8 (De Waard)

**a**



**b**

